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(54) Title: HEIGHT-RELATED GENE

(57) Abstract: The application relates to isolated regions and genes from the Y chromosome which encompass the Y specific growth gene GCY. Probes and primers are also provided.

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Height-related Gene

The sex-related height difference in humans is thought to be caused mainly by two components: first, a hormonal component determined by the sex dimorphism of bioactive gonadal steroids and second, a genetic component attributed to a Y-specific growth gene, termed GCY (Tanner, et al. 1966; Smith, et al. 1985; Ogata and Matsuo, 1992). Despite extensive mapping attempts for this gene on the human Y chromosome (Ogata, et al. 1995, Salo, et al. 1995, Rousseaux-Prevost, et al. 1996, De Rosa, et al. 1997), its precise position remains unknown. Recent evidence shows that inappropriate cytogenetic methodology in the characterization of Y-chromosomal terminal deletions has brought about some of the difficulties in elucidating the GCY-critical region. In order to overcome these problems, the inventors have considered only patients presenting *de novo* interstitial deletions for the GCY analysis on the Y chromosome (Kirsch, et al. 2000). This approach allows the assignment of GCY to a particular chromosomal interval without excluding the presence of X0-mosaicism and/or i(Yp) and idic(Yq11) chromosomes in patients with terminal deletions.

The direct comparison of overlapping interstitial deletions in seven adult males with normal height, one male with borderline height, and one patient with a large interstitial deletion and short stature resulted in the confirmation of the GCY critical interval between markers DYZ3 and DYS11. This region roughly encompasses 1.6-1.7 Mb of genomic DNA. To improve the resolution in the region of interest close to the centromere, the inventors have established additional new STS markers specific for this part of the chromosome using our bacterial artificial chromosome (BAC)/P1-derived artificial chromosome (PAC) contig. Molecular deletion analysis using these new Y-chromosomal STSs allowed the inventors to narrow down the critical interval to a genomic region of 700 kb.

Preferably the regions are to the exclusion of the regions of chromosomes on each side of the defined regions.

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Preferably the region is between SKY1 and sY83. It may include one or both the SKY1 and the sY83 regions. Preferably the region is between SKY8 and sY83 (preferably includes one or both of the SKY8 and sY83 regions), or SKY1 and SKY4.

The invention provides an isolated region of the Y chromosome between DYZ3 and DYS11 which encompasses GCY. Preferably the Y chromosome is a human Y chromosome.

The preferred region is between sY79 and sY81, preferably to the exclusion of the region of the Y chromosome outside that area of the chromosome.

Primers for use in GCY studies are also provided.

The invention further provides isolated gene/pseudogene sequences which contributes the sex related height difference in humans. These may be one or more of the gene or pseudogene sequences identified in one or more of the figures.

The invention further encompasses proteins having the same function as GCY protein and which have greater than 65% homology, greater than 70% homology, greater than 75% homology, greater than 80% homology, greater than 85% homology, preferably greater than 90% homology, and most preferably greater than 95% homology to the GCY protein. Preferably this has GCY gene activity, for example it has an effect on the height of a male mammal when expressed in that mammal.

Primers for use in detecting or amplifying a region of GCY are also provided. They may be labelled using radioactive or non-radioactive labels known in the art and used using well known methods. These methods include PCR, Southern or Northern blotting.

Experimental evidence will now be described in detail with reference to the figures in which:

Table 1 is a comparison of the adult height of patients and their siblings.

Table 2 is a table of new Y chromosomal STSs

Table 3 is the PCR/restriction digest analysis of sequence family variants in the AZFc region

Table 4 is a summary of BAC and PAC clones identified during physical map creation.

Table 5 is a summary of the genomic primers that will be used for microdeletion screening in adult males with idiopathic short stature.

Table 6 is a summary of the sequences of the isolated exon trap clones

Table 7A is a summary of primer pairs for predicted genes,

7B is a summary of primer pairs specific for the Y-copy of Adlican (ADLY),

7C is summary of RT-PCR primer sequences for ADLY,

Table 8 is RT-PCR primer sequences for exon trap clones,

Tables 9a & b are tables showing homology of exons between ADLX and ADLY.

Table 10 is a summary of sequence divergence of genes/pseudogenes from the GCY region and their homology.

Figure 1. Deletion mapping on the long arm of the human Y chromosome.
A diagram of the human Y chromosome with Yp telomere to the left and Yq telomere to the right is presented at the top. Shown below are the results of low-resolution analysis of Y-chromosomes of adult males with normal height or short stature. Along the top border, 95 Y-chromosomal STSs are listed. Except for SKY3 and SKY8 (see Table 2 for detail), all other STSs were previously reported (Vollrath et al., 1992, Jones et al., 1994, Reijo et al., 1995). Blank spaces or grey boxes indicate inferred absence or presence of markers for which assay was not performed. Asterisks indicate markers in the respective breakpoint

regions which could not be tested. In all cases where previously published data of the patients were re-investigated, the identical DNA sample used for the primary analysis was studied. (Please note that the proximal as well as the distal breakpoint of the interstitial deletion of patient #293 resides within satellite type II sequences.)

Figure 2. Sequence family variant (SFV) typing in the human DAZ locus in distal Yq11.23.

A. Overview and amplicon structure of the human Y chromosome in the vicinity of the human DAZ cluster. Each amplicon is represented by specific bands (A, B, D, E, X). Shown above are arrows indicating the orientation of each member of an amplicon family with respect to each other. The amplicon indicated by bands X arose from a portion of chromosome 1 that was transposed to the distal end of the DAZ cluster and partially duplicated.

B. Precise position of selected Y-specific STSs and the SFVs according to the physical map of the human Y chromosome. Marker sY157 is highlighted as it was suspected to be present in only one copy by multiplex PCR analysis (see text for detail).

C. Summary of STS and SFV analysis in patients with Y- chromosomal rearrangements within the human DAZ cluster region. Grey boxes indicate inferred absence or presence of markers.

D. Sequence family variant typing of SKY10 and SKY12 in genomic DNA of patient #1972. Assay is described in Table 3. Along the right are listed fragment sizes (in bp). Products are separated by electrophoresis in 3% NuSieve agarose (3:1) and visualized by ethidium bromide staining.

Figure 3. Schematic representation of the organization of the long arm pericentromeric region of the human Y chromosome

A. Diagram showing the distribution of major tandem repeat blocks and general organization of sequence homologies. Basically, the region can be subdivided in three distinct intervals: a proximal region characterized by 5bp satellite sequences (G), a central region with high homology to chromosome 1 (O), and a distal region composed of X/Y-homologous sequences (B). Below the precise position of the newly established and previously published STS markers in this region are illustrated. At the bottom border, the

PAC/BAC contig constructed with the aid of the new STS markers is shown. Prefixes RP1, 5 indicate PAC clones and RP11 BAC clones, respectively.

B. Localization of the GCY critical interval as defined by high-resolution STS mapping in patients with short stature and normal height. Black boxes indicate the presence, white boxes the absence of the respective STS. Striped boxes depict the dosage unknown regions where the breakpoint resides.

Figure 4. Molecular characterization of the GCY critical region ***a.*** Schematic illustration of the deletions in the two most crucial patients. SKY1 and sY83 demarcate its boundaries because clone Y0308 was found to have a different deletion (see Fig. 3) marking SKY1 as one of its boundaries. The AZFa region distally adjacent to the GCY region is indicated. ***b.*** Structural compartmentalization in three segments with distinct homologies. The segment composed of 5bp repeats is shown in green, the segment homologous to chromosomal subinterval 1q43 in orange, and the segment homologous to Xp22 in blue. ***c.*** Detailed description of annotated BAC clones sourcing the genomic sequence of the GCY region. ***d.*** Precise positioning of PAC clones used as substrates for exon amplification. ***e.*** Location of all exon trap clones. Due to its small size and limited single-copy content contained within exon trap clone eta1 was not amenable for further experimental analyses ***f.*** Documentation of all *in silico* generated data sets in subsequent layers: gene models (orientation; exon/intron structure) - apparent pseudogenes (exon/intron structure; orientation) - promoters. Orientation of gene models can be deduced by colour (red: orientation towards the centromere; blue: orientation towards the telomere). Please note that the chromosomal region covered by CITB-144J01, CITB-298B15, and CITB-203M13 was already intensively studied in Sargent et al. 1999.

Figure 5. Homology comparisons between genes/pseudogenes of the GCY region and their functional progenitors. Precise location of the Y-chromosomal copies is indicated. Gene pair-specific homology and subchromosomal location of the actual structural gene is shown in blue.

Figure 6. Evolutionary history of KIAA1470. On the left, chromosomal movements are illustrated. The upper lateral bar shows the exon/intron structure of the functional

progenitor in 1p36. Successive degenerating events have shaped KIAA1470 into the two pseudogenes on 1q43 and Yq11. Both copies on 1q43 and Yq11 share a 98% nucleotide sequence homology with each other, the highest among the 12 retroposons of the KIAA1470 gene family. They show 77% and 79% homology to the master gene.

Figure 7. Comparison of the structural features of the X- and Y-specific adlican gene/pseudogene. The coding exons of ADLX are illustrated as boxes. Corresponding putative exons of ADLY (also presented as boxes) were identified by homology searches. Major rearrangements in the putative transcriptional unit of ADLY are highlighted as black triangles. Sizes of mRNAs and ORF are presented for ADLX and ADLY. Primers used in RT-PCR assays are shown at their respective locations. Identical colouring above and below the separation line indicate non-selectivity for both transcripts. RT-PCR primers exclusively presented below the line are specific for ADLY.

Materials and Methods

Defining the GCY critical region

Selection of patients

Patients #293, JOLAR, #28, #63 and #95 have been described clinically in detail elsewhere (Skare et al. 1990; Ma et al. 1993; Foresta et al. 1998; Kleiman et al. 1999). Patient Y0308 corresponds to case 1 in the study of Pryor et al. 1997. Patients T.M., #1947 and #1972 are phenotypically normal males suffering from idiopathic infertility. Genomic DNA samples were extracted from peripheral blood leukocytes (#28, #63, #95, Y0308, T.M., #1947, #1972) or from lymphoblastoid cell lines (#293, JOLAR). DNA isolated from peripheral blood leukocytes of normal males and females served as internal controls.

Height assessment

As all individuals are of diverse ethnic origins, height was compared to the respective national height standards (Table 1). Patients were of similar age range. When possible, special attention was given to adult height comparisons between parents and siblings. Data are summarized along with the height standard deviation score (SDS) in Table 1. To

calculate the SDS, mean adult height and the standard deviation were taken from the corresponding national physical growth studies.

PCR analysis

Reactions were performed in a total volume of 50µl (75mM Tris/HCl pH9.0, 20mM $(NH_4)_2SO_4$, 0.1%(w/v) Tween20, 1.5mM $MgCl_2$) containing 1.0mM of each oligonucleotide primer, 100ng genomic DNA as template, 5 units of Taq DNA polymerase (Eurogentec), and each dNTP at 1mM in a thermocycler (MJ Research, Inc.) as follows: After an initial denaturation step of 95°C for 5min, samples were subjected to 30 cycles consisting of 30sec at 94°C, 30sec at 60°C and 1min at 72°C followed by a final extension step of 5min at 72°C. The Multiplex PCR was carried out as described in Henegariu et al. 1994 with minor modifications. *Alu-Alu* PCR reactions were essentially carried out as described in Nelson et al. 1991. Amplification products smaller than 1 kb were resolved on 3% NuSieve agarose/1%SeaKem GTG agarose (FMC) in 1 x TBE (0.089 M Tris-borate/0.089 M boric acid/20mM EDTA, pH 8.0). For amplification products larger than 1 kb as well as products from *Alu-Alu*-PCR, 1.5% SeaKem GTG agarose gels in 1 x TBE were used for separation.

PCR primers

Y-specific STSs, loci and PCR conditions have been described previously (Vollrath et al. 1992; Jones et al. 1994; Reijo et al. 1995). Sequences of new Y-chromosomal STSs are listed in Table 2. Y-specific STSs termed SKY were either derived from YAC, BAC and PAC end sequences or from clone-internal sequences amplified by various combinations of *Alu* primers. Primers for the markers SKY10, 11, 12, and 13 were designed to amplify fragments spanning unique restriction sites within the genomic DAZ locus (SKY10 from RP11-487K20 (AC024067), RP11-70G12 (AC006983), RP11-141N04 (AC008272), RP11-366C06 (AC015973), RP11-560I18 (AC053522), RP11-175B09 (AL359453), SKY11 and SKY12 from RP11-245K04 (AC007965), RP11-100J21 (AC017005), RP11-506M09 (AC016752), RP11-589P14 (AC025246) and SKY13 from RP11-100J21 (AC017005), RP11-589P14 (AC025246), RP11-823D08 (AC073649), RP11-251M08 (AC010682), RP11-978G18 (AC073893)) in order to detect 'sequence family variants' (SFVs).

Restriction analysis of PCR products

PCR products were resolved on agarose gels, the appropriate gel bands cut out and the DNA isolated with GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Inc.) according to the manufacturer's protocol. Fragments amplified from SKY5 and SKY6 were digested with TaqI and BsmI, respectively. To detect SFVs at SKY10, SKY11, SKY12 and SKY13, PCR products were digested with restriction enzymes as listed in Table 3.

Sequencing of BAC/PAC/YAC end fragments

DNA from BAC/PAC clones selected for end sequencing were purified with the Nucleobond PC100 Kit (Macherey-Nagel) according to the manufacturer's instructions. End fragments were directly sequenced using the Thermosequenase Fluorescent Labelled Primer Cycle Sequencing Kit (Pharmacia) and analyzed on a Pharmacia A.L.F. express (Amersham Pharmacia Biotech). YAC end fragments were generated with Alu/Vector-polymerase chain reaction and subcloned in pCR2.1 with the TOPO-TA cloning Kit (Invitrogen). Sequencing was performed as described.

Fluorescence in situ hybridization

Metaphase spreads were obtained either from primary blood samples or immortalized cell lines. Preparations were made according to standard protocols (Lichter and Cremer 1992). Cosmid and plasmid DNA was labeled by nick translation with biotin-16-dUTP (La Roche). Slides carrying metaphase spreads were kept in 70% ethanol at 4°C for one week. 200-300ng of labeled plasmid or cosmid DNA, 20-30μg of human Cot-1 DNA (GIBCO BRL), and hybridization buffer (50% formamide, 10% dextran sulfate, and 2 x SSC, pH 7.0) were mixed, denatured for 5min at 75°C and pre-annealed for 30min at 37°C. The slides were denatured for 2 min in 70% formamide and 2 x SSC, pH7.0, at 72°C (Ried et al. 1992). The pre-annealed probe was hybridized overnight in a humidifying chamber at 37°C. Slides were washed and stained with avidin-conjugated fluorescein isothiocyanate (FITC). The signal was amplified with biotinylated anti-avidin followed by staining with avidin-FITC. For the probe all human telomeres (Oncor) the instructions supplied by the manufacturer were followed. Chromosomes were counterstained with

4',6-diamidino-2-phenylindol dihydrochloride (DAPI). Images were taken separately by using a cooled charge coupled device camera system (Photometrics, Tucson AZ, USA). A Macintosh Quadra 900 was used for camera control and digital image acquisition in the 'TIF' format using the software package Nu200 2.0 (Photometrics). Separate gray scale fluorescence images were recorded for each fluorochrome. Images were overlaid electronically and further processed using the Adobe Photoshop software.

Searching the stature gene

Microdeletion screening

Exon amplification

Shotgun subcloning of PAC clones into pSPL3B. Genomic DNA from chromosome Y specific PAC clones was partially digested with *Sau3AI*. 100ng of isolated fragments in the range of 4-10Kb were ligated with 100ng of pSPL3B that had been *BamHI* digested and dephosphorylated. The ligation reaction was transformed into supercompetent E.coli XI-1 blue cells (Stratagene) and aliquots of each transformation plated on selective medium (ampicillin). Resulting colonies were subsequently pooled for plasmid DNA isolation.

Cell culture and electroporation. COS7 cells were propagated in DME medium supplemented with 10% heat inactivated calf serum. For transfections COS7 cells in between the 5th and 15th passage were grown to about 75% confluence, trypsinized, collected by centrifugation and washed in ice-cold Dulbecco's PBS. $4x10^9$ cells were then resuspended in cold 0.7ml Dulbecco's PBS and combined in a precooled electroporation cuvette (0.4cm chamber, BioRad) with 0.1ml Dulbecco's PBS containing 15µg DNA. After 10min on ice, cells were gently resuspended, electroporated (1.2kV, 25µf) in a BioRad Gene Pulser 2 and placed on ice again. After 10min cells were transferred to a tissue culture dish (100mm) containing 10ml prewarmed, CO_2 preequilibrated culture medium.

RNA isolation, RT-PCR and cloning. Cytoplasmic RNA was isolated 72hrs post transfection (QIAGEN RNeasy Kit) and first strand synthesis was performed as recommended by the manufacturer with minor modifications: 5µg of RNA was added to a solution containing 10mM of each dNTP and 2µM of oligonucleotide SA2. The mixture was heated to 65°C for 5min and then placed on ice for at least a further minute. After adding a reaction mixture containing 10x PCR buffer (Perkin-Elmer Cetus), 25mM $MgCl_2$, 0.1M DTT and RNAsin (35U/µl) , the reverse transcription reaction was transferred to 42°C for 2min. 1µl of SuperScript II RT (200U/µl; Gibco BRL) was then added and the reaction incubated at 42°C for 90min and 50°C for 30min. The entire cDNA synthesis reaction was then converted to double strand DNA using a limited number of PCR amplification cycles in the following 100µl reaction mixture: 1x PCR buffer (Perkin-Elmer Cetus), 1.5mM $MgCl_2$, 200µM dNTPs, 1µM SA2, 1µM SD6 and 2.5U Taq polymerase (Perkin-Elmer Cetus). 6 amplification cycles were used and consisted of 1min at 94°C, 1min at 60°C and 5min at 72°C. To eliminate vector-only and false positive products, 50U of BstXI (New England Biolabs) was added directly to the reactions, followed by overnight incubation at 55°C.

10µl of the digest was then used in a second PCR amplification using internal primers in the following 100µl reaction mixture: 1xPCR buffer (Perkin-Elmer Cetus), 1.5mM $MgCl_2$, 200µM dNTPs, 1µM $(CAU)_4$-SD2, 1µM $(CUA)_4$-SA4 and 2.5U Taq polymerase (Perkin-Elmer Cetus). 25 amplification cycles were used and consisted of 1min at 94°C, 1min at 60°C and 3min at 72°C. Products were separated by electrophoresis and fragments larger than the pure SD2/SA4 RT-PCR product excised and subcloned (CloneAmp pAMP1 System; Gibco BRL) into pAMP1 according to the manufacturer's protocol. Ligation reactions were then transformed in ultracompetent E.coli XL-2 blue (Stratagene) and plated on selective medium containing X-Gal/IPTG.

Identification of candidate exons. All white colonies were picked and transferred to 384-well microtiter plates containing selective medium and incubated overnight at 37°C. With a 384-pin transfer device 24.5x24.5cm culture plates with and without positively charged nylon membranes (Amersham) on top of them were inoculated and also incubated overnight at 37°C. Colonies grown on culture plates were pooled for plasmid preparation,

colonies on nylon membranes were used for colony lifts. Plasmid inserts were excised, purified, and hybridized to nylon membranes containing EcoRI-digests of the PAC clones used as the original substrate. Highlighting bands were subsequently isolated and hybridized to colony lifts to identify candidate exons. Candidate exons were isolated and sequenced by Sequitherm EXCEL II DNA Sequencing Kit (Epicentre Technologies). Sequences were automatically analyzed and read on an ALFExpress DNA sequencer. Table 6 lists the sequences of the isolated exon trap clones.

Exon Trapping. DNA from chromosome Y specific PAC (P1-derived artificial chromosome) clones RP1-148J07, RP5-1160A12, RP1-301P22, RP4-532I07 and RP1-114A11 was partially digested with *Sau3AI* and fragments in the range of 4-10Kb were individually subcloned into pSPL3B. COS7 cells were transfected and after 72hrs cytoplasmic RNA was harvested using QIAGEN RNeasy Kit. cDNA synthesis was performed as recommended by the manufacturer (Gibco-BRL). Primers flanking the cloning sites were used to identify products larger than the pure SD2/SA4 RT-PCR product. These fragments were excised, subcloned (CloneAmp pAMP1 System; Gibco BRL) into pAMP1 and sequenced. Exon trap clones were labelled with ^{32}P-dCTP by random priming and used as hybridization probes on Southern blots. Hybridization: 16 hrs at 65°C in standard hybridization buffer (Singh and Jones 1984). Wash: three times for 20 min each at 65°C in 0.1xSSC, 0.1% SDS.

In silico gene prediction. Completed genomic sequences from BAC clones RP11-75F05, RP11-461H06, RP11-333E09, RP11-558M10, CITB-298B15 and CITB-144J01 were analyzed for homologies to known genes and virtual gene content using the NIX (http://menu.hgmp.mrc.ac.uk) and Rummage (http://gen100.imb-jena.de) software packages. Computational identification of promoters and first exons was achieved by submitting BAC sequences to FirstEF (http://www.cshl.org/mzhanglab).

Reverse-transcribed polyA$^+$-RNAs and cDNA libraries. Human polyA$^+$-RNA of 16 fetal and adult tissues was purchased either from Clontech or Invitrogen. Human polyA$^+$-RNAs from 3 osteosarcoma and 1 bone marrow fibroblast cell line were isolated by the QIAGEN Oligotex kit. First-strand cDNA synthesis was essentially carried out as described (Rao et

al. 1997). Fourteen cDNA libraries were obtained either from Clontech or Stratagene. A collection of 40 cDNA libraries was also provided by the Resource Center of the German Human Genome Project (RZPD). The complete list is available on request.

Characterization of potential transcription units. After homology comparison and open reading frame (ORF) analysis of exon trap clones, primers were designed for RT-PCR amplification. Sequences are summarized in Table 8. In those cases where exon trap clones consisted of only one exon, two exon-specific primers were combined with cDNA-library specific primers in semi-nested PCR. Primers were designed from predicted gene models to amplify across exon/intron boundaries. To provide evidence of transcription, primers were used to screen a panel of cDNA libraries and polyA$^+$-RNAs (see above). In the case of potential coamplification from homologous transcripts, primers flanked Y-specific restriction sites.

Evolutionary strata classification. Sequence divergence between genes/pseudogenes of the GCY region and their functional/non-functional progenitors was determined according to Li, 1993. Sequences for all pseudogenes were extracted from genomic sequences: KIAA1470PY from BAC clone RP11-75F05 (AC011293), KIAA1470P1 from BAC clone RP11-498M14 (AL445675), ADLY from BAC clone RP11-333E09 (AC011302), ARSFP and RPS24P1 from BAC clone CITB-144J01 (AC004772), RPS24PX from BAC clone RP11-418N20 (AC119620), ASSP6 from BAC clone RP11-461H06 (AC012502) and ASSP4 from BAC clone GS1-536K07 (AC004616). Sequences for all other genes were obtained from published cDNAs, whose GenBank accesion numbers are as follows: ADLX (AF245505), ARSF (XM_035467), RPS24 (NM_033022), ASS (X01630), KIAA1470 (AB040903). THC604695PY was not analyzed as only part of its most terminal exon (consisting almost entirely of 3'UTR) was available for comparison with the X-chromosomal EST cluster (AA662182 and AA662138).

Results

Mapping of interstitial deletions

We studied the DNA of nine adult males which originally consulted reproduction centers about idiopathic infertility, but were otherwise generally healthy. Of the 9 males, 7 were unremarkable with respect to adult height. One patient, #293, with a height of 157cm, presented short stature (SDS -2.9) and one, Y0308, with a height of 165.5cm showed borderline height, being at the 3rd percentile of normal U.S. height standard (SDS -1.7). Adult height of his parents and siblings are in the normal range (Table 1), his brother being 20.5cm taller than the patient. Compared to his target height (178cm) and target range (169-187cm) he can be considered short. All men were ascertained solely on the basis of the occurrence of large *de novo* interstitial deletions on the Y chromosome. Only two of those patients had undergone previous chromosomal studies.

In our effort to localize the GCY locus, we focused on that part of the Y chromosome long arm, which was delimited by the boundaries of the interstitial deletions of the patients with short stature (Fig. 1). Recently, a detailed physical map of the human Y chromosome incorporating 758 ordered STSs and 199 completely sequenced BAC clones has been constructed (Tilford et al. 2001). We used a slightly modified PCR multiplex system (Henegariu et al. 1994) to test the absence or presence of 28 DNA loci from the Y chromosome long arm. In patients where sufficient DNA was available for further PCR analysis additional STSs were tested. As a result, 8 of 9 interstitial deletion breakpoints could be positioned (Fig. 1). As the deletions of patients JOLAR, #28, #63, #95, T.M., and #1947, all with normal height, overlap, most of the long arm of the Y chromosome could be excluded as a critical region for GCY.

As the distal breakpoint of the deletion of patient #1972 does not reside within the specific part of the Y chromosome long arm, the nature of the deletion (terminal or interstitial) remained unclear. There was also no overlap of his deletion with the deletions of patients #1947 and T.M. Relying solely on the results obtained by the STS-based interstitial deletion mapping strategy, one could not formally exclude the region distal to sY158 as a potential critical region for GCY. However, multiplex PCR analysis always showed a less

intense amplification product for STS sY157 (a Y-derived marker in close vicinity of sY158). To address this problem, the rearranged Y chromosome of patient #1972 was investigated in more detail.

Fluorescence in situ hybridization and sequence family variant typing of patient #1972

The overall integrity of the Y chromosome from patient #1972 was demonstrated by FISH of the cosmids LLOYNC03"M"34F05 (PAR1) and LLOYNC03"M"49B02 (PAR2) as well as the Y-centromere-specific probe Y-97 and the telomere-specific probe 'all human telomeres' (data not shown). Being aware of the complex structural organization of the human DAZ locus (Fig. 2A), we specifically searched for sequence family variants (SFVs). To prevent misjudging sequence errors as single nucleotide differences, PCR/restriction-digestion assays were developed only from SFVs present in at least two overlapping BAC clones. The localization of these SFVs is shown in Fig. 2B. As these SFVs could represent allelic variants, ten unrelated normal German males were typed. In all cases, the expected fragment pattern could be detected for the Y-chromosome derived sequences. In contrast, the fragment pattern deduced from the genomic sequence of the chromosome 1-derived BAC clone RP11-560I18 could not be confirmed (see Table 3 for detail). Each SFV-specific PCR/restriction digestion was compared to the presence/absence in the corresponding BAC clones.

Typing the genomic DNA of patient #1972 for all four sequence family variants (SKY10/Tsp509I, SKY11/NlaIII, SKY12/MseI, and SKY13/Cac8I + TfiI) revealed the absence of one Y-derived non-allelic sequence variant (Table 3 and Fig. 2C,D). In the case of SKY10 the distal copy is deleted. Not surprisingly, in all other typing experiments the more proximal copy of the respective SFVs was shown to be deleted.

Next, we investigated these SFVs in the two patients with the most distal breakpoints (#95 and #1947). Using genomic DNAs, we determined that both non-allelic variants of SKY11, SKY12, and SKY13 and one non-allelic variant of SKY10 were absent in patient #1947, whereas for all tested SFVs one non-allelic variant was absent in patient #95.

Taken together, these results provide evidence that the proximal breakpoint of the interstitial deletion present in the Y chromosome of patient #1972 resides within the interstitial deletion of patient #1947, thereby excluding this genomic region as a potential critical interval for GCY.

Refinement of the GCY critical interval

Based on the molecular analysis of the pericentric region of the long arm of the human Y chromosome (Williams and Tyler-Smith 1997), the physical extension of the GCY critical region as defined by the markers sY78 (DYZ3) and sY83 (DYS11) was estimated to constitute 1.6-1.7 Mb (Fig. 3A) of DNA. The most proximal 400 kb of this region consist exclusively of 5bp satellite sequences separated from the Y centromere only by *Alu* sequences. This constant part of the human Y chromosome is therefore unlikely to contain coding sequences. The remainder of the GCY critical region is composed of X/Y-homologous as well as autosomal/Y-homologous sequence blocks. At the onset of this study, only limited coverage in YAC clones was available for this region. In order to refine the GCY critical interval and to generate gene finding substrates, it was necessary to establish a BAC/PAC-contig of this region.

We generated 25 additional markers mainly by sequencing the end fragments of BAC, PAC, and YAC clones as well as clone-internal sequences amplified by various combinations of *Alu-Alu* oligonucleotide primer pairs. Of those, only 7 turned out to be Y-specific (SKY1, SKY2, and SKY4-8) (see Table 2 for detail). The BAC and PAC clones identified during the generation of the physical map are summarized in Table 4. Meanwhile, some of these clones have been completely sequenced as they form part of a tiling path for sequencing the human Y chromosome (Tilford et al. 2001). The proximal part of the cloned region between markers sY78 and SKY6 has not been sequenced to date. A selection of clones covering the entire GCY critical region is depicted in Fig. 3.

Confirming the overlap between BAC RP11-295P22 and BAC RP11-322K23 appeared to be the most crucial step in the process of contig construction. Y-specific markers derived from the opposite end fragments of both clones were suspected to amplify identical-sized

fragments from two different loci within the same 5bp satellite region. By testing several restriction enzymes known to cut frequently within 5bp satellites composed of the consensus sequence $(TGGAA)_n$, we developed loci-specific PCR/restriction digestion assays. Typing all BAC clones mapping to this sequence block with the appropriate PCR/restriction digestion assay allowed us to precisely position them thereby confirming their overlaps.

In order to narrow down the critical interval for the GCY gene, we tested for the presence of the newly generated STS in patients #293, Y0308, and JOLAR. These results allowed us to define a small region for the GCY gene (Fig. 3 and Fig. 4). Direct sequence comparison showed that the sequenced BAC clones RP11-322K23, RP11-75F05, RP11-461H06, RP11-333E09, RP11-558M10, CITB-298B15, and CITB-203M13 completely cover the mapped region between Y-STSs SKY8 and sY83 (DYS11), suggesting that it encompasses roughly 700 kb. Basically, the region can be subdivided in three distinct intervals: a proximal region characterized by 5bp repeats, a central region with high homology to chromosome 1, and a distal region composed of X/Y-homologous sequences. As the most distal part of the GCY critical region (beginning with bp1 of BAC clone CITB-144J01) was already subject of extensive research during the process of characterization of the AZFa critical region and was shown to harbour no functional gene (Sargent, et al. 1999), it was excluded from further detailed genomic DNA analysis. The most proximal part of the GCY critical region consists exclusively of satellite type 3 sequences of the 5bp consensus $(TGGAA)_n$ and is therefore also not assumed to contain any gene. Leaving these two regions out of consideration, we were able to concentrate our efforts to a smaller interval of 420 kb of DNA. Large-scale sequence comparisons performed by the Advanced PipMaker software showed no integration of Y-specific sequences into the chromosome 1 and/or chromosome X-homologous regions.

We have also established new Y-specific markers scattered uniformly across the entire 420Kb of DNA (Tab 5).

Exon trapping in the GCY critical region.

The boundaries of GCY region are defined by two deletion patients, JOLAR and Y0308 (Fig. 3). PAC clone, RP1-148J07, extends into a genomic segment exclusively composed of 5bp repeats of the satellite 3 type. The very distal PAC clone, RP1-83D22, was not included in the experimental analysis, as the region distal to sY82 was previously analyzed in the course of defining the transcriptional potential of the AZFa region (Sargent et al. 1999). To identify transcripts that might encode GCY, we used 5 PAC clones from the GCY region as substrates for exon trapping (RP1-148J07 up to RP1-114A11, Fig. 4). Each of the 5 PAC clones from the GCY region was individually subcloned and subjected to exon trapping. Nucleotide sequencing of trapped products identified 9 different exon trap clones, two of them were composed of two exons (Fig. 4, Tab. 6). All exon trap clones were isolated in several copies. Exon/intron boundaries of all 11 putative exons matches the splice site consensus. Trapped products that mapped to the GCY region were verified using PCR by their presence versus absence in males and females and GCY-deleted males with short stature. All exon trap clones revealed only one male-specific fragment on Southern blots.

In silico analysis of annotated BAC clones.

We analysed the genomic sequence of the complete GCY region using the gene prediction programs assembled by the NIX and Rummage software packages. Homologous sequences were also analysed in the non-redundant (nr) database of GenBank using the BLASTN or FASTA algorithm. BAC RP11-75F05, for example, includes a 1Kb segment with a 77% homology to the transcriptional unit KIAA1470 on chromosome 1p36 (Fig. 5). On BAC RP11-461H06 and CITB-144J01, for example, sequences of 2.5 and 1Kb length showed a 88% and 81% homology with the genes ASS and RPS24 on chromosome 9q34 and 10q22, respectively. The Y-chromosomal copies ASSP6 and RPS24P1, however, represent pseudogenes and have a progenitor on Xp22 that has been translocated to the Y chromosome. Two pseudogenes on RP11-333E09 and CITB-144J01, THC604695PY and ARSFP, represent deleted copies of Xp22 specific genes.

BAC RP11-333E09 includes a deleted duplication (ADLY) of the adlican gene on chromosome Xp22 (ADLX). ADLX has been previously shown to be upregulated in osteoarthritic tissue and therefore likely plays a role in bone metabolism. The Y

chromosome copy, therefore, constitutes an important candidate for a gene involved in growth. Despite the loss of exons 3 and 4 as a consequence of intrachromosomal recombination, its basic structural organization (Fig. 7) and sequence homology to ADLX (Tab. 9a) could still allow to encode a functional protein with similar molecular properties. This observation was enforced by a unified predicted gene model of ADLY by all gene-finding programs (cf1; Fig. 4). Taking the functionality of the predicted ADLY promoter for granted and assuming ADLY would start at the ATG codon also used on the X chromosome, an in-frame stop codon at position +359 would result in premature termination. One additional promotor was predicted in the sense strand of the last intron of ADLY. There is, however, no obvious correlation between the promoter position and the significance for potential ADLY expression.

Using various gene-finding programs we detected 17 gene models in the GCY region (Fig. 4f). Only five (ar1, cf1, cr1) overlapped with transcriptional units identified by homology search. Conceptual translations of 14 models revealed no protein matches. With respect to location and orientation promoters predicted by FirstEF could be assigned to KIAA1470P, ADLY, RPS24P1, and ARSFP.

In conclusion, there is no identity of exon trap clones and gene models/homologies or pseudogenes KIAA1470PY, ASSP6, and THC604695PY. Considering ADLY as the most attractive candidate for the GCY locus, we directly compared the exon/intron boundaries of the Y- and X-derived copy (Tab. 9b). Exons 3 and 4 of ADLX are deleted on the Y copy. The remaining 3 internal exons still possess correct 5' and 3' splice sites.

Searching for a transcriptional unit.

Homology searches performed with all exon trap clones and predicted gene models against the dbEST segment of GenBank did not yield any Y-specific EST. PCR and PCR/restriction digestion assays with primers corresponding to all putative transcriptional units were carried out. Primers derived from all exons of ADLY (Tab. 7B, 7C), the most prominent GCY candidate, were used to screen reverse-transcribed polyA$^+$-RNAs from osteosarcoma and bone marrow fibroblast cell lines. Whereas ADLX was shown to be expressed in all tested cell lines (with the exception of neuronal tissues), no ADLY specific

specific transcript was detectable. More extensive screening of polyA$^+$-RNAs from various adult and fetal tissues basically led to the same result. We also tested all putative transcriptional units in the GCY region for expression in polyA$^+$-RNAs from 21 tissues and 49 cDNA libraries. RT-PCR assays did not provide proof of a transcribed gene.

Evolutionary features of the GCY critical region.

High sequence homology of the Y chromosome to other chromosomal regions is consistent with an evolutionarily recent transposition of those regions to the Y chromosome. More subtle nuances in synonymous nucleotide divergences of homologous gene pairs (K_s) allow their integration into distinct evolutionary strata, group 1-4 (Lahn and Page 1999). The calculated K_s values for all gene pairs in the GCY region along with K_s values from reference genes of the different stratas are given in table 6. We noted that the K_s values for all X-Y gene pairs can be grouped into the most recent evolutionary stratum (group 4), having been embarked on X-Y differentiation 30 to 50 million years ago. This classification is independent of the actual functional state of X-chromosomal genes. Comparing K_s values between the Y-copies in the GCY region and their functional progenitors clearly demonstrates that decay of the X-chromosomal copies took place before the X-Y recombination occurred. Even more prominent is the difference between K_s values for the chromosome 1 - chromosome Y gene pairs. The low K_s value for the KIAA1470P1/KIAA1470PY gene pair points towards a very recent transposition to the human Y (Fig. 5). Supporting evidence comes from fluorescent *in situ* hybridization in primates delineating this event to a time period of about 5 to 6 million years ago (Wimmer et al. 2002). The K_s value for the comparison of KIAA1470PY with its functional progenitor in 1p36 date the underlying intrachromosomal transposition roughly to about 150-170 million years ago.

As the frequency of nonsynonymous substitutions (K_a) is a function of both evolutionary time and selective constraints on the encoded proteins, the degree of constraint can be reflected in the ratio K_s/K_a (Li, 1993): Values greater than one indicate the presence of constraints on both homologs, and values in the vicinity of one are consistent with lack of constraint on at least one homolog. All determined K_s/K_a ratios suggest that natural selection on the Y copies is not ongoing thereby underlining their pseudogene status.

We searched the nr database of Genbank with the homology transitions and the distal border of the GCY region to precisely determine the physical extent of the homologous regions on chromosomal subintervals 1q43 and Xp22. To identify highly conserved segments, we used Advanced PipMaker (Schwartz et al. 2000, http://bio.ces.psu.edu) for comparing the corresponding DNA. Inspection of the compound dot plot allows the identification of those portions of the GCY region absent in homologous sequences. As the overall homology of Y/1 and Y/X in conserved regions is already in the range of 94-97% and 96-99%, putative protein-coding exons are not expected to show average percent identities higher than the non-coding environment. Careful dot plot analysis showed that all novel sequences that have accumulated in the GCY region on the Y after the separation from its autosomal or X-chromosomal counterpart are exclusively of repetitive origin. Particularly evident is the prevailing preponderance of integrated LINEs family members.

Discussion

Since the issue on the existence of a Y-specific growth gene (GCY) was first raised, there have been several attempts to define its precise location. Whereas initial studies unanimously pointed towards a common region of the Y chromosome long arm (Salo et al. 1995), more recent investigations have led to the identification of two non-overlapping critical intervals (Rousseaux-Prevost et al. 1996, Ogata et al. 1995, De Rosa et al. 1997). FISH analyses resolved this apparent contradiction by presenting clear evidence that the patient materials used in these initial investigations contained 45,X0 cells and/or i(Yp) or idic (Yq11) chromosomes (Kirsch et al. 2000). Both genetic parameters influence the adult height of a given individual, thereby rendering it impossible to predict whether such patients have lost GCY or not. Studies with patients carrying *de novo* interstitial deletions are, therefore, much better suited to address the problem of GCY localization.

In the course of winnowing the literature for patients with small interstitial deletions, in particular close to the centromere, it became clear that those patients are very rare. This prompted us to extend our search for patients carrying large *de novo* interstitial deletions, irrespective of their actual adult height. We examined 9 adult patients, 7 of whom

presented normal height. Futhermore, we could show overlapping deletions, thereby excluding GCY to reside between the Y-specific marker DYS11 and the pseudoautosomal region 2 (PAR2). Two patients, #293 and Y0308, presented interstitial deletions enabling the restriction of the GCY critical region to approximately 700 kb of DNA. This region is therefore predicted to harbour one or more genes required for normal human growth.

Exon amplification and gene modeling in the GCY region.

Although much attention has been drawn to the various azoospermia (AZF) critical regions in Yq11 as well as Y-encoded testis-specific or ubiquitously expressed genes, the GCY region up to now was not searched systematically for transcription units. We have used exon amplification, homology search, and *in silico* gene prediction to identify putative genes within this region. This information now provides the means to test candidate genes for involvement in human linear growth regulation. Up to date, the major problem in defining the GCY gene was the lack of potential transcription units assigned to this portion of the human Y chromosome. Prior to this study, there were only two pseudogenes, RPS24P1 and ARSFP, that mapped to the GCY critical region (Sargent et al. 1999).

By exon amplification we isolated 9 different exon trap clones, two of which were composed of two exons. Parallel sequencing efforts of the GCY region by the Human Genome Project allowed us to complete our catalog of potential transcription units in the GCY region. No Y-specific ESTs were assigned to the region. The Nix and Rummage software programs were used to analyze sequence data of completed BACs to predict potential genes in the sequence. We have identified 4 new genes/pseudogenes and 17 gene models. Of the 17 gene models, only five have homologies to the identified genes/pseudogenes. A gene model homologous to ADLY (cf1) was uniformly predicted by all gene-finding programs. Though, the probability given by various gene finding programs might be overestimated with regard to the gene model cf1. Very large exons, as present in ADLY, are less likely to be predicted correctly, but they are most unlikely to be completely missed. Consequently the tendency to classify actual pseudogenes as functional genes increases with the presence of large exons. The failure to trap exons of the putative ADLY transcription unit, albeit possessing correct splice sites, might be an intrinsic feature of Y-chromosomal sequences. Complete representation of the AZFc region in cosmid/P1

clones used for exon-trapping experiments (Reijo et al. 1995) led to the detection of DAZ as the only gene out of a possible 8 genes/gene families located in this region (Kuroda-Kawaguchi et al. 2001).

Surprisingly, we observed no concordance between the gene models and the exon trap clones. It is possible that exon amplification is dependent on the presence of functional splice sites in the genomic sequence whereas gene modeling is mainly based upon the in-phase hexamer measure (Rogic et al. 2001), a method determining the incidence of oligonucleotides of length six in a specific open reading frame. On the other hand, the prediction of correct splice sites is less important since such signal sensors have low information content and are usually degenerate. Consequently, the exon trap clones need not to be necessarily part of one of the predicted gene models, although a substantial fraction of the trapped exons (7/11) are composed of 75 to 200 nucleotides, a length range in which exons are most accurately predicted. Likewise, the putative exons assembled to a distinct gene model do not necessarily represent real exons.

It is possible that the eventual number of genes in the GCY region is smaller since exon trap clones and/or gene models turn out to be part of the same transcripts or do not represent genes at all. Despite the number of potential transcription units in the region, however, the search for the critical one might still be complicated by the fact that the phenotypic effect caused by mutation of the GCY locus is hard to be defined precisely. This makes it difficult to predict an expression profile, especially when the gene function is unknown. Since human linear growth is a multifactorial trait, growth failure is quite common.. Although at least nine growth-controlling genes have been identified up to now, only few cases present disease-causing mutations within those genes. Definition of the transcription units in the region should now facilitate mutation studies, especially since full-length genes/pseudogenes have been isolated.

Although reverse-transcribed polyA$^+$-RNAs and cDNA libraries have been extensively screened, we have not detected any transcript specific to the Y. This raises the question whether our approach was suitable. To assess its usefulness we have verified the expression pattern of 20 genes known to be essential for bone development at GenePage

(http://genome-www5.stanford.edu). At least double presence for each selected gene was warranted by our screening efforts. This corroborates the existence of an unusual gene with an extremely confined spatial and/or temporal expression pattern.

Evolutionary features as a clue to the GCY locus?

To gain more insight into the molecular genesis of the GCY critical region, we used two methods. First, we validated the functional state of the genes/pseudogenes within the GCY region by comparing them with their direct and functional progenitors. All gene pairs showed K_s/K_a ratios of 1 to 2 rather indicating that the Y copy is a pseudogene. This result assigns the X-Y gene pairs to evolutionary stratum 4 which fits very well since all those gene pairs share a common evolutionary history. Only one gene pair out of this class, AMELX/Y, still encodes a functional X- and Y-copy (Salido et al. 1992). The Y-copy of KIAA1470 clearly could be classified as a pseudogene by comparing it with its functional progenitor on 1p36. Second, we made use of large-scale sequence comparison in order to identify potential differences between the subintervals of the GCY region and their homologous counterparts in Xp22 and 1q43. Neither subregions with a conservation level above the molecular environment nor small genomic fragments newly integrated into the GCY critical region could be detected. Furthermore, promoter prediction carried out simultaneously on homologous genomic sequences revealed no differences. This clearly excludes substantial rearrangements within the GCY critical region and lends support to a gene underlying male-specific regulatory mechanisms.

Table 1 Adult height comparison of patients and their siblings

Case	Country of origin	Height of patient (cm) and standard deviation score	National height standard (cm)	Heights of family members (cm) and standard deviation score
#293	U.S.A.	157 (SDS -2.9) short	176.9 (SD 6.8)	(F) 170 (M) normal (B) normal
Y0308	U.S.A.	165.5 (SDS -1.7) borderline (short?)	176.9 (SD 6.8)	(F) 170 (M) 168 (B) 188 (SDS +1.7) (S) 170 (SDS -0.4)
JOLAR	United Kingdom	168 (SDS -1.0) normal	174.7 (SD 6.7)	(F) normal (M) normal (B) normal
#28	Italy	175 (SDS -0.3) normal	176.7 (SD 6.5)	(F) normal (M) normal
#63	Ethiopia	170 (SDS +0.3) normal	168.0 (SD 7.4)	(F) normal (M) normal
#95	Israel	185 (SDS +1.4) normal	175.6 (SD 6.8)	(F) normal (M) normal
T.M.	Belgium	182 (SDS +1.3) normal	173.5 (SD 6.7)	(F) normal (M) normal
#1947	Germany	175 (SDS -0.8) normal	179.9 (SD 6.4)	(F) normal (M) normal
#1972	Germany	181 (SDS +0.2) normal	179.9 (SD 6.4)	175 (F) 165 (M) 172 (S) (SDS +1.0)

The standard deviation score (SDS) was calculated based on the equation: SDS = (X-M)/SD, where X is an individual's adult height and M and SD are the mean adult height and the ± 1 standard deviation of the normal population, respectively.

(M) mother, (F) father, (S) sister, (B) brother, (NA) not available.

Table 2 Y-chromosomal STSs

STS	Left Primer	Right Primer	Product
SKY1	GGACATTTGGCTGCAGAGAT	TGGCAATGCACTCTCATCAT	255
SKY2	TCAGGACAGACAGGCTGCTA	CCTGCCACTGAGCTCCTTAC	~1700
SKY3	TTCTCCCTCATCTTCCAAGC	GCTTCCATCCATTAGCAAGG	167
SKY4	CCTTTCATTCCATTCTCTTCCA	CGCACTTTATGGACTGCAA	111
SKY5*	CCCTCGTCCATTTCTTTTGA	CCTCGAATTTAATGGATTGC	202
SKY6*	TCAATGGATGCACAGTGTGGC	TCCACTGAATTCCATTGCAC	328
SKY7	GGGAGTGCAAAGGGAAAGAT	CTTTCCATGGGGTGACATTC	223
SKY8	CCATTCATTCGAGTTCATTACG	ATTGGAATGGAATCGGACAG	189
SKY9	GGCCGATGGTCAAACTGTTA	GAAACGGGCTCTGAAATTCT	531
SKY10*	ATAAGGGGCAGGTTTGTCAC	GCTACTTATTCAGTGTTTAACTGACAC	329
SKY11*	AAAGTGGGTGAAGGACATGG	TTTTTGTTTGTGGCAGGTG	469
SKY12*	TTGAGTCACTGGGGATAACTG	TATGGCCCACAATCACTTCA	216
SKY13*	GGCAGCCTAGAAAGTCTTGTTC	CCCTTGGGATTTTGTCTGTT	198

Markers indicated with a * amplify DNA fragments from more than one genomic locus (see Chapter *Restriction analysis of PCR products* for detail).

Table 3 PCR/Restriction Digest Analysis of Sequence Family Variants in the AZFc

STS	Restriction enzyme	BAC clones	Fragment sizes (bp) after restriction	STS	Restriction enzyme	BAC clones	Fragment sizes (bp) after restriction
SKY10	Tsp509I	487K20	279,50	SKY12	MseI	245K04	88,57,39,32
		70G12	329			506M09	145,39,32
		560I18	329*				
				SKY13	Cac8I/TfiI	100J21	97,83,23
SKY11	NlaIII	245K04	217,154,79,19			589P14	175,23
		506M09	233,221,15			251M08	97,50,33,23

*The submitted sequence of the chromosome 1-derived BAC clone RP11-560I18 (AC053522) does not show a Tsp509I restriction site within the genomic fragment amplified by the primer pair SKY10. Restriction analysis of fragments amplified from male and female genomic DNA, from a somatic cell hybrid line containing chromosome 1 as the only chromosome of human origin and from the BAC RP11-560I18 as well shows two fragments of ~180bp and ~155bp indicating a sequence error in the complete sequence of the BAC clone.

Table 4 Summary of BAC and PAC clones identified during physical map creation

Y-STSs	Positive BACs (RPCI11)	Positive PACs (RPCI1, 3-5)
sY83	not screened	83D22
sY82	not screened	83D22, 114A11, 157G08, 966C15
GY8	not screened	114A11, 168E21, 271D03, 635F21, 765H16, 806O15, 904E13, 966C15
sY81	not screened	301P22, 1079J08, 1078C20, 1160A12
14A3C*	not screened	148J07, 1136A14, 1160A12, 1196I23
sY79	75F05, 79E14, 102G24, 322K23, 417D23, 600D11, 612E10, 725I12, 863I08, 903M02, 1125H21	1149H11
SKY1	376B16, 544C11, 544M21	56A05, 85D24, 958M03
SKY2	79P12, 295P22, 376L20, 828O24, 886I11, 910C06	829H08
SKY4	75F05, 322K23, 612E10	not screened
SKY5	174I24, 271E18, 295P22, 588E18, 620J20, 632F11, 684H19, 705O19	not screened
SKY6	174I24, 271E18, 295P22, 588E18, 620J20, 632F11, 684H19, 705O19	not screened

* 14A3C is a hybridization probe previously described by Tyler-Smith et al. 1993. It detects a Y-specific HindIII-fragment of 3.5 kb and an additional autosomal fragment.

Table 5 Genomic primer pairs for microdeletion screening in adult males with idiopathic short stature

Primersequence (5'→ 3')				genomic location*	
forward	reverse	product size	primer	forward	reverse
ATTTCCACCGAAACCCATTT	CTCCCCTACCACCAACACAC	251	A72	72300-72318	72549-72530
AGGGCCCTCACATGATTAAA	GCGACACCATTTCTTTCCAT	255	A92	91949-91968	92204-92185
GACATCGTGGTGTCTGTTGC	CAGACGTTGTTCAGGTCGTG	232	A111	111509-111528	111740-111721
GCACCATTAGTGCGCTTGT	TTCTCCCTTTACCCCAAATTC	269	A134	134542-134560	134810-134790
CCAGCAGGAGTCTTGGAGTC	TGAGAGGCACCTACGGTTAGA	250	A158	157911-157930	158160-158140
CCAAGCATGCCTTCCTAAAG	TGCCTTCTCATCTGCTTGTG	147	B17	17598-17617	17744-17725
ATCCTGGGAGATGCATCAGA b_r_002for	TGAGTCCTAAACCGTACACATACA	209	B37	37406-37425	37614-37591
CAATGGAAATGTTGCAGGTG	TCCTGCCCTGCTGTTAGAGT	158	B59	59871-59890	60028-60009
GCAAGGGTGTTGCAAGTTTA	TGCATATTGTCCACACATGG	360	B82	82128-82147	82487-82468
AAAGAGAAGGGCCCTGTGAT	CTAGGCAACAGCACTGGAAA	239	B102	102854-102873	103092-103073
AAAATCCAACTTCCCCAGTG	GCAAGAATCTGGGCTCTCAC e_f_001rev	353	C17	17307-17326	17659-17640
CACTGGGGAAGGCTGTGATA	CATTGTCATCACTGCCAGGT	339	C37	37271-37290	37609-37590
CCCACTTCTTCTCCAAAGTCC c_r_005rev	GCACCCGTTTTCCTGATCTA	139	C56	56159-56179	56297-56278
GGGGCATATTCTACACACCAA	TGAAATGGCAAACCTTTCAGA et_c_003rev	495	C77	76731-76751	77225-77205
AAGAATGGAAGGATCTCCAAGA	TCTGTGCAGAAATGATGGATTC	342	C97	96759-96780	97100-97079
TGGTAGTGGGAAACTGCTCA c_r_003rev	TGGTGTGCTAAGTGGCTGTC	144	C120	120709-120728	120852-120833
GCTGCAGTTAGCTAAACCAAGAC	ATTCTGCCTGAACCTCCAGA	162	C142	142289-142311	142450-142431

Table 6 Sequences of isolated exon trap clones

Exon trap clones:

Name	Sequence (5'→3')	Size (bp)	Orientation
et_a_001	GGTCTTTGGCTCAACTCAGGTTCCCTCTACCTGAAATGATCCACCTTCAGAGAATTGGATG	61	reverse
et_a_002	CTGTGTTGCCTCCTCGATGGGAAAAGAAACAAGCGCACTAATGGTGCATTT (exon 1) CTGGAGCATCAGGGGTGTCTTCTATGATCAAGGAAGGAAGCCACTCAGGGTGATAGAGCTGCAGACTTCTGCTTGGTCA CTCTGATAGCTCTGGGAACACTGTGCACCTCTCTGGCTGTGATGGGGAAACT (exon 2)	182	reverse
et_a_003	CTTTTACATAGAATGGTAACTCCTTTTGCACCTCGTGTTTTTTC	44	forward
et_a_004	AAAGTTGGTAGTTCGCTCCCGGGCTGATGCTCAGAGTGTGGAACTTGAGGAGCTGCGGTGACATCCTGCAGCCACACGG GAGGTGGCTCCTCAGGGGCGATTGCTGGCTGTGTCACCACCAGGGGACACCGGGCACAGCTTGAAGCTTGGGGACAGGG AGCTGAGAGGCAC	171	reverse
et_c_001	GATTACATGGACTACTATATTTAAAATTCCTTCTAAACTTTTTCCCATTTCTGCTCAATTTTCATTCTCCAATATTTGC AAAACTTAAAGTTC	93	forward
et_c_002	GCTGAACATTATTTCTTTATTCCAGATTAGAGGACTAGGATTCATGGGATTATGCATCAA	60	forward
et_c_003	GGAAATCTTGAAATGGCAAACCTTTCAGAAGAGATGGCAGAGACTCTCCTACATATTCTGTTCTCAAT	68	reverse
et_c_004	ACACTGGAAGAATTGGTGTCTAGGCAGTCTGGGATAATAGCCTAGTTCTAAGGACATTATCATTGATCCCTTTATAGGC CATAGACCTCCAT (exon 1) TTCTTCCTGTTGGTGCAGGAGGGTGATTAAGGGCTTTTCCTACCTTAAGTTGATCAAAGTGGTATTTTCATAAGATTAA TCTGGCAGCAGAATGCA (exon 2)	188	reverse
et_c_005	CTTGGTTGGGAAAATATGGCCACCATATTGCTGGGAAAGCCACCAAGAGTGGACTGTTACCAATATCCAAGGGACATGA	79	forard

P706097GB

Table 7A Primer pairs for predicted genes

Primer pairs for predicted genes					genomic location[3]	
forward	reverse	product size[1]	predicted gene	restriction enzyme[2]	forward	reverse
GCTTGGAACTTGAGGTGCTC	GGAGATGTGGGCTTGTGAGT	482	a_r_001		104600-104581	103332-103351
CTGTGGGTGCATTAGGTGTG	CTGGTACATGCTGCCTGCT	841	a_r_002		144939-144920	111361-111379
GACCTCTTTTGAGAAAGTCAGCA	AAAGCAATGGCAACAAAAGC	446	b_f_001		30214-30236	61274-61255
AGAGGGAGGAAAGAGCCATC	GTTGTACGGGCTGCAGAATC	790	b_r_001		25244-25225	762-781
TGAGTCCTAAACCGTACACATACA	TTTCTGTGCGTGAGAACACA	122	b_r_002		37614-37591	29995-30014
TCTCTGTGGTGCTGATCCTG	GCAAGAATCTGGGCTCTCAC	730	c_f_001		6243-6262	17659-17640
ATCCCTATTCGCCCCTTAGA			c_f_001b		10734-10753	
ACCTCAGGGTGCAGCTTTTA	TGAGCAGTTTCCCACTACCA	350	c_f_002	Bsh1236I	80230-80249	120728-120709
GCTGCAGTTAGCTAAACCAAGAC	TTCTGCAAGGGTCTGGTTCT	123	c_f_003	AlwI	142289-142311	162171-162152
CACAGAAGCCAGGGATCG	GCATCTCGCCCTTTCCTC	1150	c_r_001	BamHI	6361-6344	2888-2905
CAACACTGTACACCGCAACA	TTCTCCAAAGTCCGATACCTG	172	c_r_002	BspMI	81022-81003	56167-56187
TGGAGACATTCACAACGTCAA	TGGTAGTGGGAAACTGCTCA	325	c_r_003	AluI	129988-129968	120709-120728
AGCTGCCTGACTTCTTGGAA	CTTGCCCACACCTTGATCTC	574	c_r_004	AccI	170431-170412	162765-162784
CGTGCTGGATTCCTATTTGG	CCCACTTCTTCTCCAAAGTCC	212	c_r_005	MspI	66318-66299	56159-56179

[1]predicted product size in bp; [2]Potential Y-derived transcript copies will be cut with the indicated restriction enzyme, potential X-derived transcripts remain uncut; [3]indicates primer positions (orientation centromer to telomer) in the predicted gene containing BAC (a, b, c or d).

Table 7B Primer pairs for Y copy of Adlican

Primersequence (5'→ 3')	Direction with respect to putative transcription orientation		primer
GACTCCTGGCCTTGACTTGA	forward		AdlYEx1
TCTCTGTGGTGCTGATCCTG	forward		cf1
GGAGGAGCAAAAACAAGAAGAGA	forward		cf1-117
ACTGATGAGCACGGGAACC	forward		cf1-205
TCCATCCTGAAAGTGCCTG	forward		C17c
ACATGTATACATGCTGCCAA	forward		C18
CAGCGAAGGAAAGCACATTT	forward		AdlYEx5
GGCGACCTGAAGGGGACT	forward		cf1-1915
CTGTCCAGTCCTCAGGAAGC	forward		C21
GAAGCATCCACCAAAGCG	forward		cf1-4679
ACAGCGGGCGCTATGAGT	forward		cf1-4a
CAGGATCAGCACCACAGAGA	reverse		AdlYEx2
CTGGGGAAGTTGGATTTTCTC	reverse		C17b
ACCAGGTTCCCGTGCTCA	reverse		cf1-227
GCAAGAATCTGGGCTCTCAC	reverse		cf1
ACTGTGATTCCCACCGTGAT	reverse		C17c
TTGTTTTGAGGAACGCCTCT	reverse		C18
GGATGTGGGATCTGGTGAG	reverse		cf1-2079
GGGTGTAATTTTCTCCCATTG	reverse		AdlYEx5
CGTCCGTTTCAGCAGTGACA	reverse		cf1-4810
CTGACGTCCGTCCTCTGC	reverse		cf1-4b
ATGGACAGTGATCCGGTTTC	reverse		cf1-6453
TGAGCTGCACGATCAACCTC	reverse		cf1-6559

Table 7C **RT-PCR primer sequences for ADLY**

Primer	Sequence (5'→ 3')	Pos. in ADLY[1]	Pos. in ADLX	ADL exon[2]
		Forward primer		
AdlYEx1	GACTCCTGGCCTTGACTTGA	44-63	--	1
cf1	TCTCTGTGGTGCTGATCCTG	184-203	184-203	2
AdlYEx5	CAGCGAAGGAAAGCACATTT	2177-2196	--	5
C21	CTGTCCAGTCCTCAGGAAGC	5089-5108	5620-5639	5
cf1-4a	ACAGCGGGCGCTATGAGT	5971-5988	6502-6519	6
		Reverse Primer		
AdlYEx2	CAGGATCAGCACCACAGAGA	203-184	203-184	2
cf1	GCAAGAATCTGGGCTCTCAC	914-895	1435-1416	5
AdlYEx5	GGGTGTAATTTTCTCCCATTG	3103-3083	--	5
cf1-4b	CTGACGTCCGTCCTCTGC	6143-6126	6631-6614	6
cf1-6453	ATGGACAGTGATCCGGTTTC	7158-7139	7649-7630	7

[1] ADLY refers to the gene predicted according to homology comparison with functional X-adlican. [2] Numbering of exons is based on the exon/intron organization of the X-copy. Please note: RT-PCR with cf1for/rev would generate different-sized products from adlican copies. cf1-4a/cf1-6453 and C21/Cf1-4b amplification products encompass chromosome-specific restriction sites (cf1-4a/cf1-6453: Y-BamHI, X-PsyI; C21/cf1-4b: Y-NlaIII, X-SacI).

Table 8 RT-PCR primer sequences for exon trap clones

Exon trap clone	Forward primer	Reverse primer
eta2	GCACCATTAGTGCGCTTGT	GAGCATCAGGGGTGTCTTCT
eta3	a: TTACATAGAATGGTAACTCCTTTTGC b: AACTCCTTTTGCACCTCGTG	
eta4		a: GCTGATGCTCAGAGTGTGGA b: GATTGCTGGCTGTGTCACC
etc1	a: TTTAAAATTCCTTCTAAACTTTTTCC b: CCCATTTCTGCTCAATTTTCA	
etc2	a: GCTGAACATTATTTCTTTATTCCAGA b: AGAGGACTAGGATTCATGGGATT	
etc3		a: TGAAATGGCAAACCTTTCAGA b: GGCAGAGACTCTCCTACATATTC
etc4	TGGCCTATAAAGGGATCAATG	GGTGCAGGAGGGTGATTAAG
etc5	a: GAAAGCCACCAAGAGTGGAC b: ACCAATATCCAAGGGACATGA	

The product size of eta2 is 175 bp and of etc4 166bp. For single exon-trap clones semi-nested PCR was carried out: a reflects the outer pimer, b the inner one.

Table 9a : Homology comparison of exons

Exon	Size (bp)		Nucleotide sequence homology (%)
	ADLX	*ADLY*	
1	127	127	85
2	215	217	97
3	129	deleted	--
4	390	deleted	--
5	4967	4958	93
6	900	944	95
7	3061	3097	94

Table 9b : Exon/intron boundaries of conserved exons

Exon	Intron/Exon		Exon/Intron	
	ADLX	*ADLY*	*ADLX*	*ADLY*
1	GAGCTGCCTC	GAGCTGCCTC	CCAAGGACAG**gt**gaggaccc	CCAAGGATAG**gt**gaggaccc
2	tctacctcag**GTATCCGAGA**	tctacctcag**GTATCCGAGA**	TCAATTTGGG**gtttgtacca**	TCAATTTGGG**gtttgtacca**
5	tttgttttag**GAATTCTGAA**	tttgttttag**GAATTCTGAA**	GTTTCCACAG**gtaatatgtt**	GTTTCCACAT**gtaagatttt**
6	ttttctccag**GAGCTCTTAT**	ttttctccag**GAGTTCTTAT**	CGCTCTTCAG**gtaggcagct**	CGCTTTTCAG**gtaggcagct**
7	ttttctgtag**TTTTGATAGC**	ttttctgtag**TTTTGATAGT**	ATATTCTCCCC	ATATTCTCCCC

Table 10 **Sequence divergence of genes/pseudogenes from the GCY region and their homologues**

Gene pair	K_s	K_a	K_s/K_a	DNA divergence	Protein divergence	Sequence compared (nt)
			Genes in GCY region			
			X/Y gene pairs			
ADLX/ADLY	0.10	0.07	1.4	8	15	1260
ARSF/ARSFP	0.09	0.08	1.1	9	18	456
RPS24PX/RPS24P1	0.16	0.09	1.8	11	22	357
RPS24/RPS24P1*	0.28	0.17	1.6	20	30	369
ASSP4/ASSP6	0.10	0.08	1.3	9	20	1230
ASS/ASSP6*	0.17	0.09	1.9	11	22	1230
			1/Y gene pairs			
KIAA1470P1/KIAA1470PY	0.05	0.03	1.7	4	7	1194
KIAA1470/KIAA1470PY*	0.34	0.18	1.9	22	35	1203
			X/Y gene pairs - Group 4			
ARSE/ARSEP	0.05	0.04	1.2	4	9	615
			X/Y gene pairs - Group 3			
DFFRX/DFFRY	0.33	0.05	6.6	11	9	7671
			X/Y gene pairs - Group 2			
SMCX/SMCY	0.52	0.08	6.5	17	15	4623
			X/Y gene pairs - Group 1			
RBMX/RBMY	0.94	0.25	3.8	29	38	1188

* If chromosome X- or 1-derived copies of genes from the GCY region were not functional, Y-copies were additionally compared with their functional progenitors.

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Claims

1. An isolated region of the Y chromosome between SKY1 and sY83 which encompasses the Y-specific growth gene GCY.

2. An isolated region according to claim 1 which is about 700 Kb in size.

3. An isolated region according to claim 1 or 2 in which the Y chromosome is a human chromosome.

4. An isolated region according to claims 1 or 3 which is between SKY8 and sY83.

5. An isolated region according to claims 1 or 3, which is between sY79 and sY81.

6. An isolated GCY protein, encoded by a region of the Y chromosome within the interval SKY1 and sY83.

7. An isolated GCY protein according to claim 6 encoded by a region within the interval SKY8 and sY83.

8. An isolated GCY protein according to claim 7 encoded by a region between sY79 and sY81.

9. An isolated GCY protein according to claim 8, which is ADLY or a functional fragment thereof.

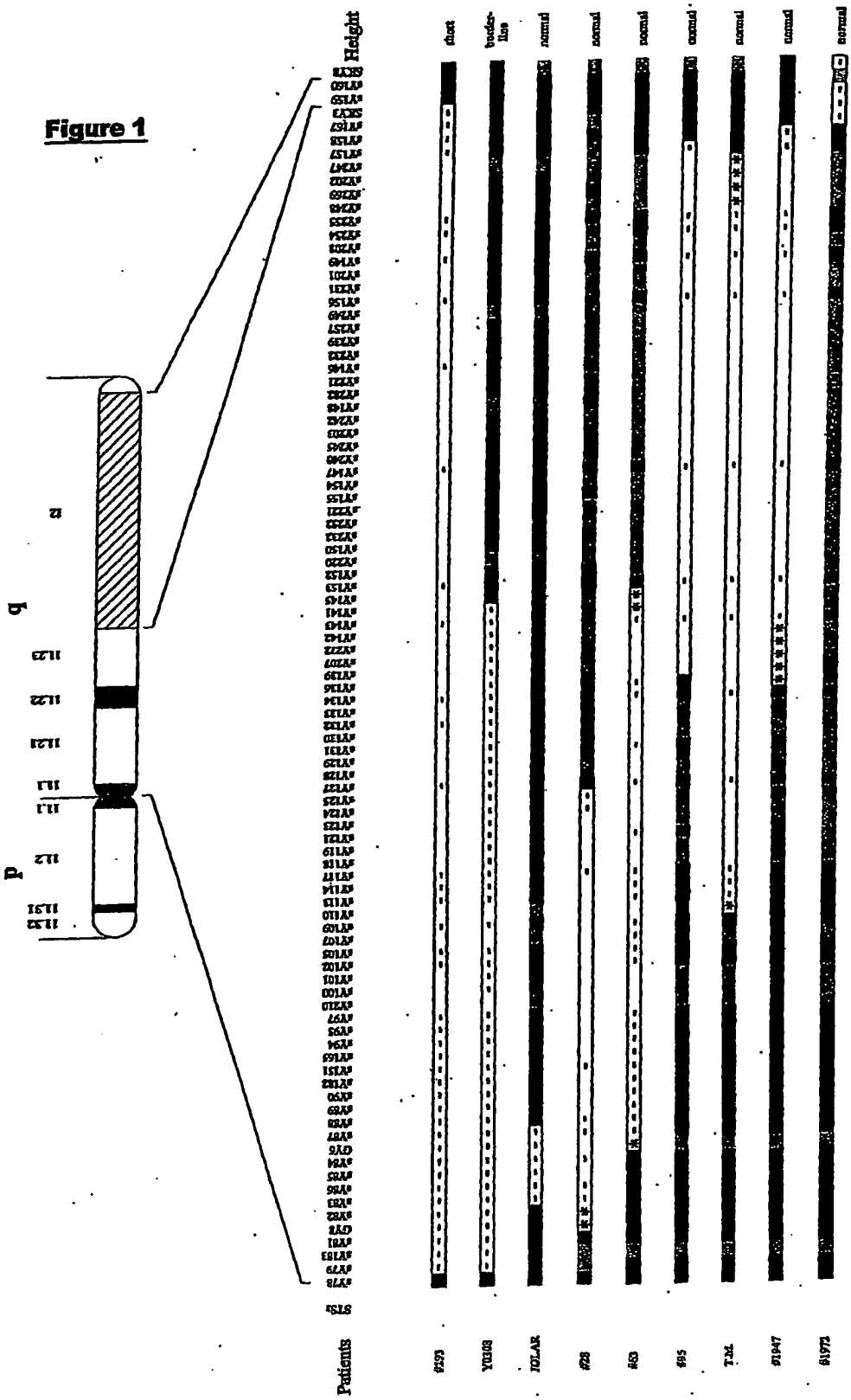
10. A nucleic acid primer having a nucleic acid sequence selected from a nucleic acid sequence as shown in Tables 2, 5, 6, 7A, 7B, 7C or 8.

11. A method of studying GCY localisation or identifying a GCY gene associated with height comprising the use of a primer according to claim 10 to selectively amplify or detect a region of a nucleic acid molecule.

12. An isolated protein having greater than 65% homology to the GCY protein of claims 6-9, and which contributes to the sex-related height difference in humans.

13. Use of a nucleic acid molecule comprising at least a portion of the isolated region of the Y chromosome between markers SKY8 and sY83, or a sequence complementary thereto, to identify the presence or absence of a GCY gene associated with height.

Figure 1


Height
Patients
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YH08
IOLAR
#28
#63
#85
T24
#1947
#1973
deletion
normal
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11.2
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12
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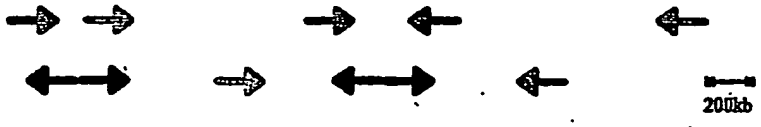
Figure 2

A



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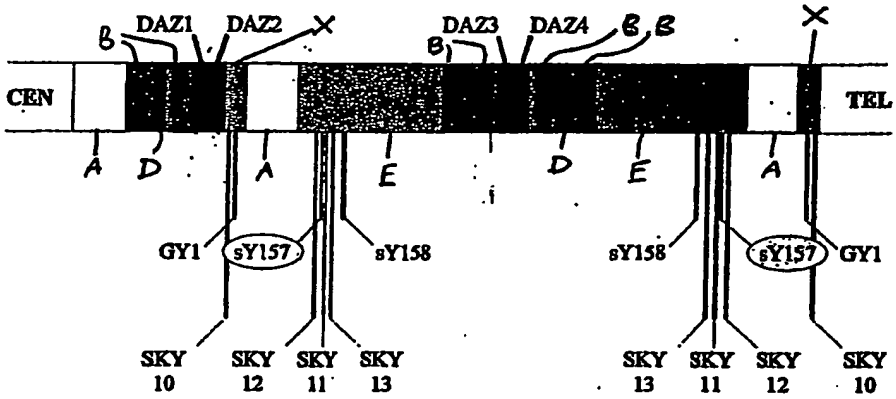

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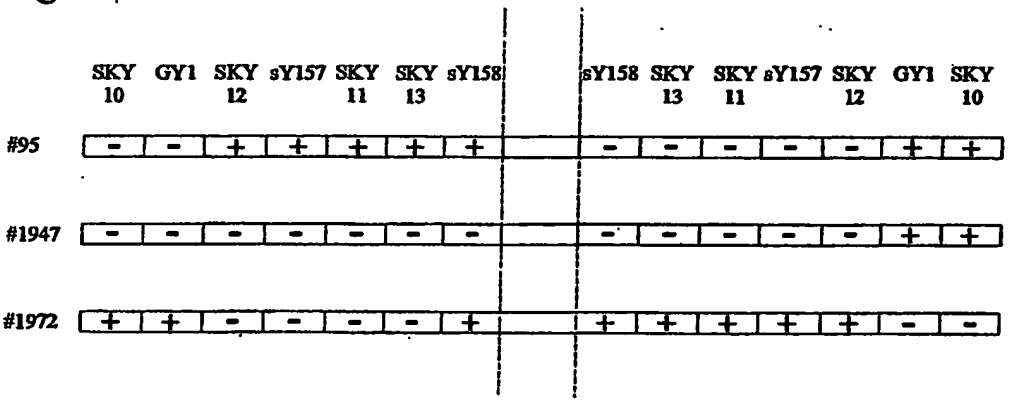
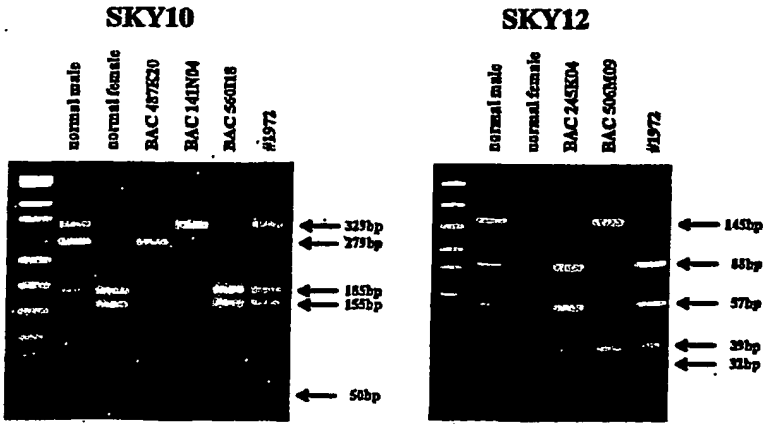


D



A

Figure 3

Homology to:

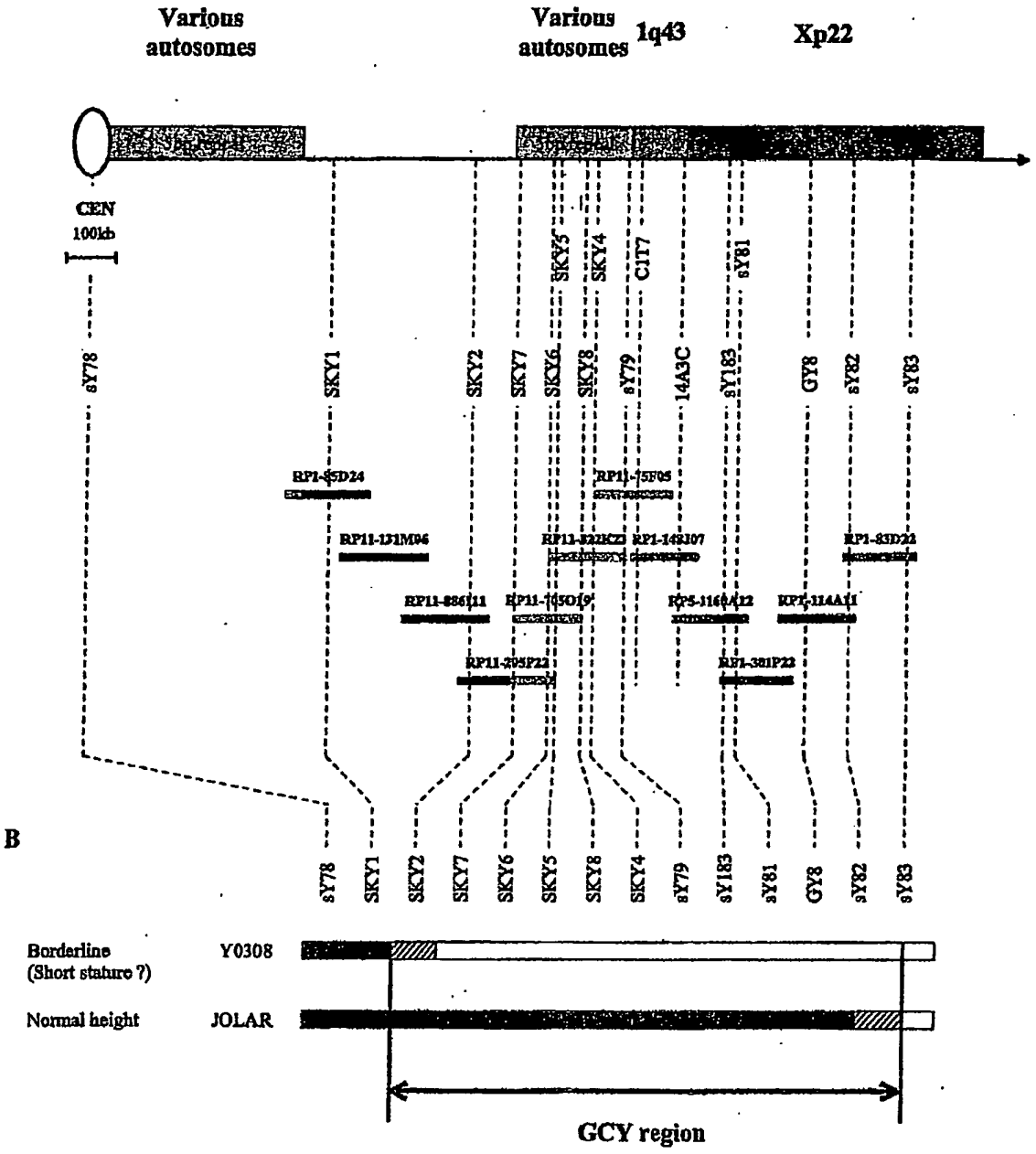


Figure 4

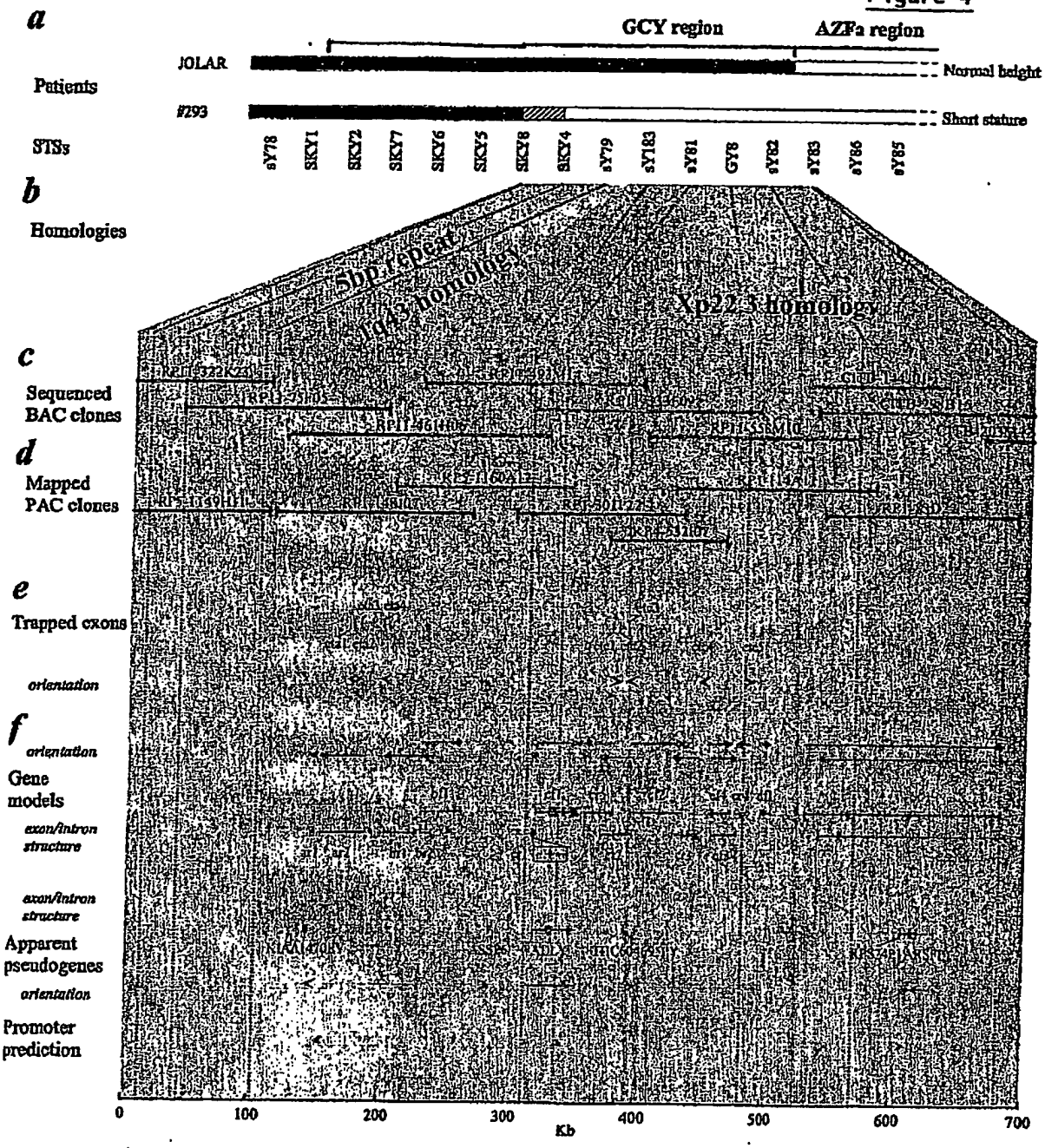

a
GCY region
AZFa region
JOLAR
Normal height
Patients
#293
Short stature
STSs
sY78
SKY1
SKY2
SKY7
SKY6
SKY5
SKY8
SKY4
sY79
sY183
sY81
GY8
sY82
sY83
sY86
sY85
b
Homologies
5bp repeat
1q43 homology
Xp22.3 homology
c
Sequenced BAC clones
d
Mapped PAC clones
e
Trapped exons
orientation
f
orientation
Gene models
exon/intron structure
exon/intron structure
Apparent pseudogenes
orientation
Promoter prediction
0
100
200
300
400
500
600
700
Kb

Figure 5

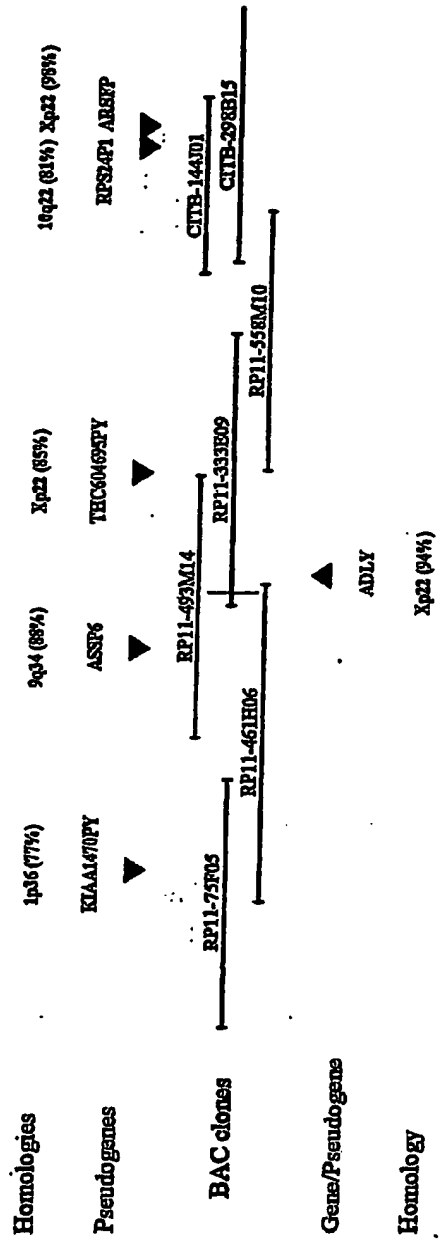

Homologies
1p36 (77%)
9q34 (88%)
Xp22 (85%)
10q22 (81%) Xp22 (98%)
Pseudogenes
KIAA1470PY
ASSP6
THC60469PY
RPS24P1 ARSFP
BAC clones
RP11-75F05
RP11-461H06
RP11-493M14
RP11-333E09
RP11-558M10
CITB-144J01
CITB-298B15
Gene/Pseudogene
ADLY
Homology
Xp22 (94%)

Figure 6

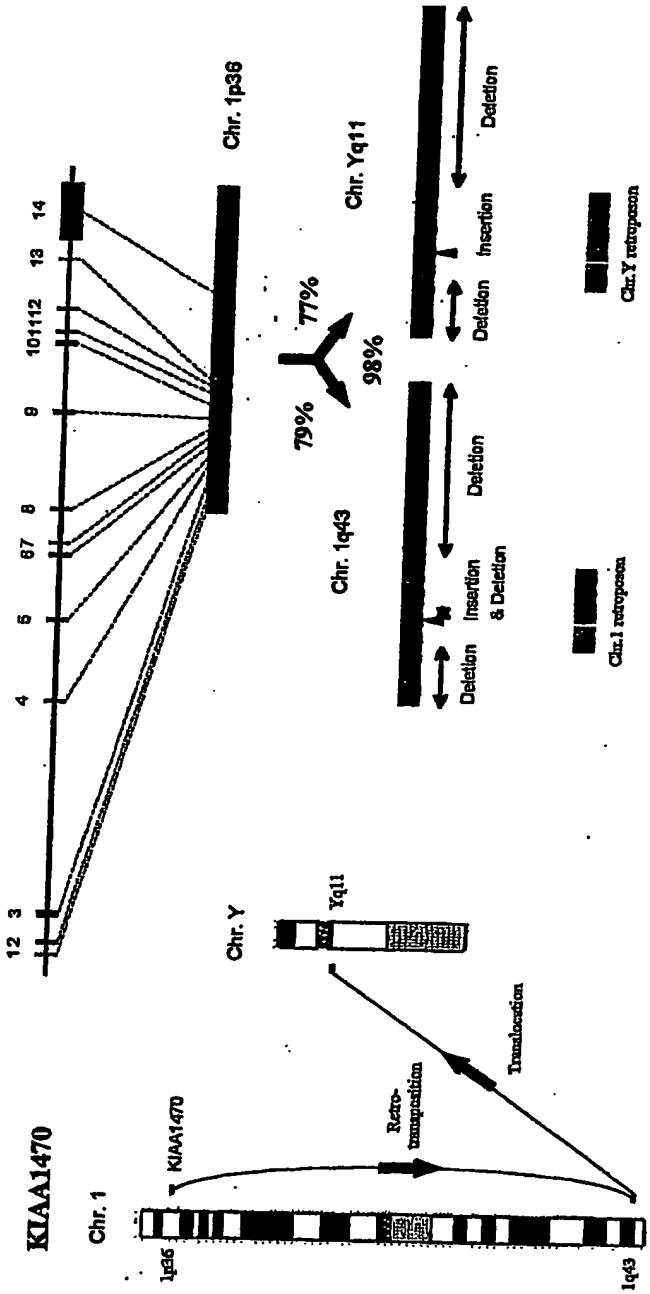
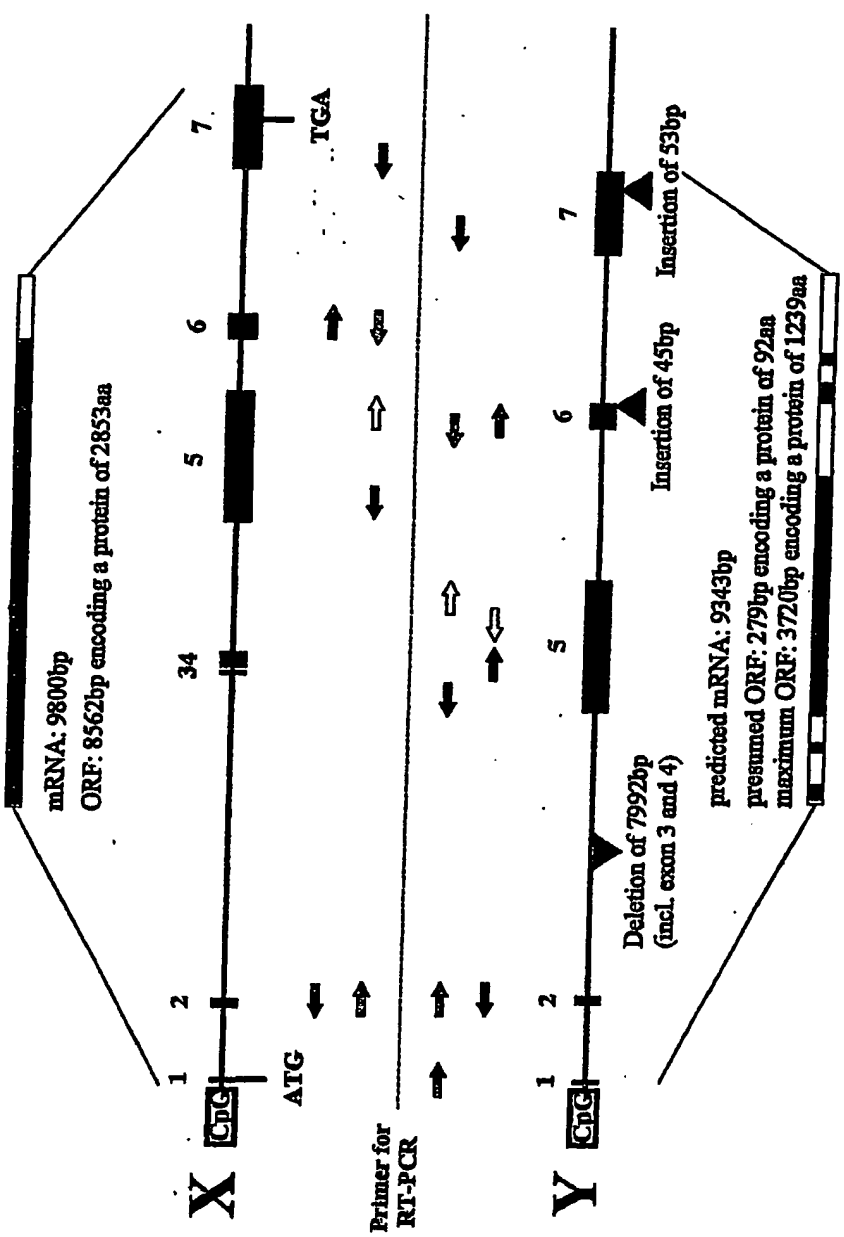

Chr. 1p36
Chr. Yq11
Deletion
Insertion
Deletion
Chr.Y retrogene
77%
98%
79%
Chr. 1q43
Deletion
Insertion & Deletion
Deletion
Chr.1 retrogene
12 3
4
5
67 8
9
101112
13
14
Chr. Y
Yq11
Translocation
Retro-transposition
KIAA1470
KIAA1470
Chr. 1
1p36
1q43

Figure 7


mRNA: 9800bp
ORF: 8562bp encoding a protein of 2853aa
ATG
TGA
CpG
X
Y
Primer for RT-PCR
Deletion of 7992bp (incl. exon 3 and 4)
Insertion of 45bp
Insertion of 53bp
predicted mRNA: 9343bp
presumed ORF: 279bp encoding a protein of 92aa
maximum ORF: 3720bp encoding a protein of 1239aa

10/511708
DT01 Rec'd PCT/PTO 15 OCT 2004

SEQUENCE LISTING

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<120> Height-related gene

<130> P706097PCT

<140> PCT/EP03/04546
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gcagacttct gcttggtcac tctgatagct ctgggaacac tgtgcacctc tctggctgtg
120

atggggaaac t
131

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120

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<213> Artificial

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60

ttcattctcc aatatttgca aaacttaaag ttc
93

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<220>
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cattgatccc tttataggcc atagacctcc at
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21

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tggagacatt cacaacgtca a
21

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<220>
<223> Primer: c_r_003 reverse

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tggtagtggg aaactgctca
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<210> 95
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<220>
<223> Primer: c_r_004 forward

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agctgcctga cttcttggaa
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<210> 96
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<220>
<223> Primer: c_r_004 reverse

<400> 96
cttgcccaca ccttgatctc
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<210> 97

<211> 20
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<220>
<223> Primer: c_r_005 forward

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cgtgctggat tcctatttgg
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<210> 98
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<220>
<223> Primer: c_r_005 reverse

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cccacttctt ctccaaagtc c
21

<210> 99
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<220>
<223> Primer: AdlYEx1 forward

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gactcctggc cttgacttga
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<210> 100
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<220>
<223> Primer: cf1 forward

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tctctgtggt gctgatcctg
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<210> 101

<211> 23
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<220>
<223> Primer: cf1-117 forward

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ggaggagcaa aaacaagaag aga
23

<210> 102
<211> 19
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<220>
<223> Primer: cf1-205 forward

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actgatgagc acgggaacc
19

<210> 103
<211> 19
<212> DNA
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<220>
<223> Primer: C17c forward

<400> 103
tccatcctga aagtgcctg
19

<210> 104
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<220>
<223> Primer: C18 forward

<400> 104
acatgtatac atgctgccaa
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<210> 105

<211> 20
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<220>
<223> Primer: AdlYEx5 forward

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cagcgaagga aagcacattt
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<210> 106
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<220>
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ggcgacctga aggggact
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<210> 107
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<220>
<223> Primer: C21 forward

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ctgtccagtc ctcaggaagc
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<210> 108
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<212> DNA
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<220>
<223> Primer: cf1-4679 forward

<400> 108
gaagcatcca ccaaagcg
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<210> 109

<211> 18
<212> DNA
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<220>
<223> Primer: cf1-4a forward

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acagcgggcg ctatgagt
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<210> 110
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<220>
<223> Primer: AdlYEx2 reverse

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caggatcagc accacagaga
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<210> 111
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<212> DNA
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<220>
<223> Primer: C17b reverse

<400> 111
ctggggaagt tggattttct c
21

<210> 112
<211> 18
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<220>
<223> Primer: cf1-227 reverse

<400> 112
accaggttcc cgtgctca
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<210> 113

<211> 20
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<220>
<223> Primer: cf1 reverse

<400> 113
gcaagaatct gggctctcac
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<210> 114
<211> 20
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<220>
<223> Primer: C17c reverse

<400> 114
actgtgattc ccaccgtgat
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<210> 115
<211> 20
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<220>
<223> Primer: C18 reverse

<400> 115
ttgttttgag gaacgcctct
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<210> 116
<211> 19
<212> DNA
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<220>
<223> Primer: cf1-2079 reverse

<400> 116
ggatgtggga tctggtgag
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<210> 117

<211> 21
<212> DNA
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<220>
<223> Primer: AdlYEx5 reverse

<400> 117
gggtgtaatt ttctcccatt g
21

<210> 118
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Primer: cf1-4810 reverse

<400> 118
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<210> 119
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<212> DNA
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<220>
<223> Primer: cf1-4b reverse

<400> 119
ctgacgtccg tcctctgc
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<210> 120
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<220>
<223> Primer: cf1-6453 reverse

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<210> 121

<211> 20
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<220>
<223> Primer: cf1-6559 reverse

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tgagctgcac gatcaacctc
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<210> 122
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<212> DNA
<213> Artificial

<220>
<223> RT-PCR Primer: AdlYEx1 forward

<400> 122
gactcctggc cttgacttga
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<210> 123
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<212> DNA
<213> Artificial

<220>
<223> RT-PCR Primer: cf1 forward

<400> 123
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<210> 124
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<212> DNA
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<220>
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<400> 124
cagcgaagga aagcacattt
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<210> 125

<211> 20
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<220>
<223> RT-PCR Primer: C21 forward

<400> 125
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<210> 126
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<220>
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<400> 126
acagcgggcg ctatgagt
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<210> 127
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<220>
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<400> 127
caggatcagc accacagaga
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<210> 128
<211> 20
<212> DNA
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<220>
<223> RT-PCR Primer: cf1 reverse

<400> 128
gcaagaatct gggctctcac
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<210> 129

<211> 21
<212> DNA
<213> Artificial

<220>
<223> RT-PCR Primer: AdlYEx5 reverse

<400> 129
gggtgtaatt ttctcccatt g
21

<210> 130
<211> 18
<212> DNA
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<220>
<223> RT-PCR Primer: cf1-4b reverse

<400> 130
ctgacgtccg tcctctgc
18

<210> 131
<211> 20
<212> DNA
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<220>
<223> RT-PCR Primer: cf1-6452 reverse

<400> 131
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<210> 132
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<212> DNA
<213> Artificial

<220>
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<400> 132
gcaccattag tgcgcttgt
19

<210> 133

<211> 20
<212> DNA
<213> Artificial

<220>
<223> RT-PCR primer for exon trap clone: eta2 reverse

<400> 133
gagcatcagg ggtgtcttct
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<210> 134
<211> 26
<212> DNA
<213> Artificial

<220>
<223> RT-PCR primer for exon trap clone: eta3a forward

<400> 134
ttacatagaa tggtaactcc ttttgc
26

<210> 135
<211> 20
<212> DNA
<213> Artificial

<220>
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<400> 135
aactcctttt gcacctcgtg
20

<210> 136
<211> 20
<212> DNA
<213> Artificial

<220>
<223> RT-PCR primer for exon trap clone: eta4a reverse

<400> 136
gctgatgctc agagtgtgga
20

<210> 137

<211> 19
<212> DNA
<213> Artificial

<220>
<223> RT-PCR primer for exon trap clone: eta4b reverse

<400> 137
gattgctggc tgtgtcacc
19

<210> 138
<211> 26
<212> DNA
<213> Artificial

<220>
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<400> 138
tttaaaattc cttctaaact ttttcc
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<210> 139
<211> 21
<212> DNA
<213> Artificial

<220>
<223> RT-PCR primer for exon trap clone: etc1b forward

<400> 139
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21

<210> 140
<211> 26
<212> DNA
<213> Artificial

<220>
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<400> 140
gctgaacatt atttctttat tccaga
26

<210> 141

<211> 23
<212> DNA
<213> Artificial

<220>
<223> RT-PCR primer for exon trap clone: etc2b forward

<400> 141
agaggactag gattcatggg att
23

<210> 142
<211> 21
<212> DNA
<213> Artificial

<220>
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<400> 142
tgaaatggca aacctttcag a
21

<210> 143
<211> 23
<212> DNA
<213> Artificial

<220>
<223> RT-PCR primer for exon trap clone: etc3b reverse

<400> 143
ggcagagact ctcctacata ttc
23

<210> 144
<211> 21
<212> DNA
<213> Artificial

<220>
<223> RT-PCR primer for exon trap clone: etc4 forward

<400> 144
tggcctataa agggatcaat g
21

<210> 145

<211> 20
<212> DNA
<213> Artificial

<220>
<223> RT-PCR primer for exon trap clone: etc4 reverse

<400> 145
ggtgcaggag ggtgattaag
20

<210> 146
<211> 20
<212> DNA
<213> Artificial

<220>
<223> RT-PCR primer for exon trap clone: etc5a forward

<400> 146
gaaagccacc aagagtggac
20

<210> 147
<211> 21
<212> DNA
<213> Artificial

<220>
<223> RT-PCR primer for exon trap clone: etc5b forward

<400> 147
accaatatcc aagggacatg a
21

<210> 148
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<212> DNA
<213> Artificial

<220>
<223> Exon 1: Intron/Exon ADLX boundary

<400> 148
gagctgcctc
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<210> 149

<211> 10
<212> DNA
<213> Artificial

<220>
<223> Exon 2: Intron/Exon ADLX boundary

<400> 149
gagctgcctc
10

<210> 150
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 2: Intron/Exon ADLX boundary

<400> 150
tctacctcag gtatccgaga
20

<210> 151
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 2: Intron/Exon ADLY boundary

<400> 151
tctacctcag gtatccgaga
20

<210> 152
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 5: Intron/Exon ADLX boundary

<400> 152
tttgttttag gaattctgaa
20

<210> 153

<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 5: Intron/Exon ADLY boundary

<400> 153
tttgttttag gaattctgaa
20

<210> 154
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 6: Intron/Exon ADLX boundary

<400> 154
ttttctccag gagctcttat
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<210> 155
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 6: Intron/Exon ADLY boundary

<400> 155
ttttctccag gagttcttat
20

<210> 156
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 7: Intron/Exon ADLX boundary

<400> 156
ttttctgtag ttttgatagc
20

<210> 157

<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 7: Intron/Exon ADLY boundary

<400> 157
ttttctgtag ttttgatagt
20

<210> 158
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 1: Exon/Intron ADLX boundary

<400> 158
ccaaggacag gtgaggaccc
20

<210> 159
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 1: Exon/Intron ADLY boundary

<400> 159
ccaaggatag gtgaggaccc
20

<210> 160
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 2: Exon/Intron ADLX boundary

<400> 160
tcaatttggg gtttgtacca
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<210> 161

<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 2: Exon/Intron ADLY boundary

<400> 161
tcaatttggg gtttgtacca
20

<210> 162
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 5: Exon/Intron ADLX boundary

<400> 162
gtttccacag gtaatatgtt
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<210> 163
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 5: Exon/Intron ADLY boundary

<400> 163
gtttccacat gtaagatttt
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<210> 164
<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 6: Exon/Intron ADLX boundary

<400> 164
cgctcttcag gtaggcagct
20

<210> 165

<211> 20
<212> DNA
<213> Artificial

<220>
<223> Exon 6: Exon/Intron ADLY boundary

<400> 165
cgcttttcag gtaggcagct
20

<210> 166
<211> 11
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<220>
<223> Exon 7: Exon/Intron ADLX boundary

<400> 166
atattctccc c
11

<210> 167
<211> 11
<212> DNA
<213> Artificial

<220>
<223> Exon 7: Exon/Intron ADLY boundary

<400> 167
atattctccc c
11